Role of Phagosomes and Major Histocompatibility Complex Class II (MHC-II) Compartment in MHC-II Antigen Processing of *Mycobacterium tuberculosis* in Human Macrophages

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*Mycobacterium tuberculosis* resides in phagosomes inside macrophages. In this study, we analyzed the kinetics and location of *M. tuberculosis* peptide-major histocompatibility complex class II (MHC-II) complexes in *M. tuberculosis*-infected human macrophages. *M. tuberculosis* peptide–MHC-II complexes were detected with polyclonal autologous *M. tuberculosis*-specific CD4+ T cells or F9A6 T hybridoma cells specific for *M. tuberculosis* antigen (Ag) 85B (96-111). Macrophages processed heat-killed *M. tuberculosis* more rapidly and efficiently than live *M. tuberculosis*. To determine where *M. tuberculosis* peptide-MHC-II complexes were formed intracellularly, macrophages incubated with heat-killed *M. tuberculosis* were homogenized, and subcellular compartments were separated on Percoll density gradients analyzed with T cells. In THP-1 cells, *M. tuberculosis* Ag 85B (96-111)–DR1 complexes appeared initially in phagosomes, followed by MHC class II compartment (MIIC) and the plasma membrane fractions. In monocye-derived macrophages, *M. tuberculosis* peptide–MHC-II complexes appeared only in MIIC fractions and subsequently on the plasma membrane. Although phagosomes from both cell types acquired lysosome-associated membrane protein 1 (LAMP-1) and MHC-II, THP-1 phagosomes that appeared only in MIIC fractions and subsequently on the plasma membrane. Although phagosomes from both cell types acquired lysosome-associated membrane protein 1 (LAMP-1) and MHC-II, THP-1 phagosomes that support formation of *M. tuberculosis* peptide–MHC-II complexes had increased levels of both LAMP-1 and MHC-II. Thus, *M. tuberculosis* phagosomes with high levels of MHC-II and LAMP-1 and MHC both the potential to form peptide–MHC-II complexes from *M. tuberculosis* antigens in human macrophages.

*Mycobacterium tuberculosis* is an intracellular pathogen that is phagocytosed by professional phagocytes such as macrophages and dendritic cells. A variety of receptors promote *M. tuberculosis* phagocytosis and these include among others the complement receptors CR1, CR3, and CR4, the mannose receptor, and the scavenger receptor class A (14, 33, 42). Following phagocytosis, *M. tuberculosis* resides inside plasma membrane-derived phagosomal vesicles. While the majority of bacterial phagosomes undergo a process of acidification and maturation, *M. tuberculosis* inhibits this process (16, 37).

CD4+ T cells are critical for control of *M. tuberculosis* infection in animals and humans (5, 10) and are activated when mycobacterial antigens are processed and presented by major histocompatibility complex class II (MHC-II) molecules. Processing of soluble antigens has been extensively studied. Newly synthesized MHC-II molecules bind invariant chain in the endoplasmic reticulum and target to endocytic compartments that function in antigen processing, e.g., the MHC class II compartment (MIIC) (1, 12, 22, 23, 30, 36, 39). Here the invariant chain is degraded, leaving only the class II-associated invariant chain peptide (CLIP) bound to MHC-II (8). CLIP is then replaced with an antigenic peptide generated from the degradation of the soluble antigen. The resulting peptide–MHC-II complexes are transported to the cell surface for presentation to CD4+ T cells.

Processing of particulate antigens by the MHC-II pathway and the specific role of phagosomes in this process are still being defined. Latex bead phagosomes were shown to contain MHC-II, degrade phagosome-associated antigen, and directly mediate peptide–MHC-II complex formation (24, 26, 28, 29). More recently, Ramachandra et al. demonstrated that *M. tuberculosis* phagosomes derived from *M. tuberculosis*-infected murine bone marrow macrophages could also mediate the formation of peptide–MHC-II complexes (27). There is also evidence suggesting that endocytic compartments play a role in the processing of certain bacteria. Following internalization of *Streptococcus pyogenes*, endocytic compartments mediate the formation of peptide–MHC-II complexes after receiving phagosome-derived antigen fragments (9). In the current studies, we have analyzed the intracellular compartments involved in the generation of *M. tuberculosis* peptide–MHC-II complexes in *M. tuberculosis*-pulsed human macrophages.

To analyze processing of *M. tuberculosis* antigens in the human macrophage cell line THP-1 and in human monocye-derived macrophages (MDMs), we have used F9A6 murine T hybridoma cells, specific for the antigen (Ag) 85B (96-111) epitope presented by HLA-DR1 or polyclonal autologous *M. tuberculosis*-specific CD4+ T cells. Ag 85B, a mycolyltransferase involved in cell wall biosynthesis (4), is a major target of human T-cell responses to *M. tuberculosis* and a leading vac-

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cine candidate (6, 15, 19, 40). The F9A6 T hybridoma cells allowed study of the processing of a defined antigen (Ag 85B), while polyclonal autologous M. tuberculosis-specific CD4+ T cells allowed analysis of processing of mycobacterial antigens in general. Processing of heat-killed M. tuberculosis by THP-1 cells and MDMs for presentation to T cells was more rapid than processing of live M. tuberculosis.

Subcellular fractionation coupled with T-cell assays demonstrated that phagosomes mediated the formation of M. tuberculosis Ag 85B (96-111)–DR1 complexes in THP-1 cells, but in MDMs M. tuberculosis peptide–MHC-II complexes were formed in MIIC but not phagosomes. Analysis of M. tuberculosis phagosomes by flow organellometry demonstrated that phagosomes derived from both THP-1 cells and MDMs acquired lysosome-associated membrane protein 1 (LAMP-1) and MHC-II, but THP-1-derived phagosomes had increased levels of both LAMP-1 and MHC-II. This suggests that MDM phagosomes may not be able to support formation of M. tuberculosis peptide-MHC complexes due to its low MHC-II levels and altered phenotype. In conclusion, M. tuberculosis peptide-MHC-II complexes can be formed in both phagosomes and MHC in macrophages.

MATERIALS AND METHODS

Bacteria. M. tuberculosis H37Ra was obtained from the American Type Culture Collection (ATCC 25117; Manassas, VA) and grown to log phase in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 1% glycerol and 10% Middlebrook albumin dextrose catalase enrichment (Difco). Bacteria were harvested and frozen at −80°C in RPMI 1640, 10% fetal bovine serum, and 6% glycerol. Bacterial concentration was determined by counting CFU on Middlebrook 7H10 agar plates (Difco). Heat-killed M. tuberculosis was prepared by incubating M. tuberculosis at 80°C for 30 min (killing was confirmed by CFU counting). Prior to infection of cells, all M. tuberculosis preparations were pelleted, washed, and declumped by 10 passages through a 26-gauge needle followed by sonication for 40 seconds at 80 cycles in an Eppendorf centrifuge for 2 min to remove clumps. To label live or heat-killed M. tuberculosis with fluoresein, 106 bacteria were pelleted, resuspended in 1 ml phosphate-buffered saline (pH 9.1) and mixed with 25 μl of 20 mg/ml FLUOS (Boehringer, Mannheim, Germany) in dimethyl sulfoxide for 10 min at room temperature. Labeled M. tuberculosis bacteria were washed twice and declumped prior to use. M. tuberculosis bacteria were incubated in medium containing 10% non-heat-treated pooled human serum with anti-biotics for 30 min at 37°C prior to incubation with cells.

Cells and media. Unless otherwise specified, all cells were cultured at 37°C in 5% CO2. The human monocyte cell line (THP-1) was obtained from the American Type Culture Collection (TIB 202) and cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 50 μg/ml mercaptoethanol, 1 mM sodium pyruvate, 2 mM l-glutamine, 10 mM HEPES buffer, nonessential amino acids, 100 μM penicillin, and 100 μg/ml streptomycin (BioWhittaker, Walkersville, MD). Cultures were maintained at a cell concentration between 2 x 106 and 106 cells/ml.

To induce differentiation of cells to macrophages, THP-1 cells were activated in medium supplemented with 10 ng/ml phorbol myristate acetate (Sigma, St. Louis, MO). After 24 h, medium was removed and cells were incubated for an additional 24 h with new medium containing 50 U/ml recombinant human gamma interferon (IFN-γ) (Endogen, Woburn, MA). Activated THP-1 cells were used in all experiments to upregulate MHC-II levels. T hybridoma cells (F9A6) specific to M. tuberculosis Ag 85B (96-111)–DR1 complexes was generated in HLA-DR1 (subtype 01) transgenic mice as described previously (7, 11). The T-cell hybridoma F9A6 was maintained in Dulbecco’s modified Eagle’s medium (BioWhittaker) supplemented as described above for THP-1 cells.

MDMs were obtained from healthy adult donors. For certain experiments HLA-DR1 and HLA-DR2 donors were selected. Heparinized blood was diluted 1:1 with RPMI and centrifuged at 300 × g for 45 min at room temperature over a Ficoll-sodium diatrizoate solution (Ficoll-Paque; Pharmacia Fine Chemicals, Inc.). The layer containing peripheral blood mononuclear cells (PBMCs) was harvested, and monocytes were enriched by plastic adherence for 1 h at 37°C. Adherent cells were purified by immunomagnetic bead separation according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). The mean purity of positively selected CD14+ monocytes was 97% as determined by flow cytometry. Monocytes were cultured in RPMI 1640, 10% pooled human serum at 37°C for 7 days. By this time, the cells had acquired macrophage morphology. Recruitment and participation of healthy blood donors in our research were approved by the University Hospitals of Cleveland and Instituto Nacional de Enfermedades Respiratorias Institutional Review Board.

Generation of antigen-specific CD4+ T cells. Polyclonal autologous CD4+ T cells were obtained from purified protein derivative-positive (PPD) donors and stimulated as described by Tan et al. (34). PBMCs (2 x 106 cells/well) were stimulated in 24-well plates with live M. tuberculosis at a multiplicity of infection of 1:10 and 50 U/ml of interleukin-2 (IL-2). The culture was additionally supplemented with IL-2 on days 3, 5, and 7. After 7 to 10 days CD4+ T cells were positively selected by immunomagnetic bead separation according to the manufacturer’s instructions (Miltenyi Biotec). The mean purity of positively selected CD4+ T cells was 96% ± 2% as determined by flow cytometry. Cells were allowed to rest for 24 h before being used in antigen processing assays.

HLA typing of donors. DNA was isolated from PBMCs by the DNeasy kit (QIAGEN) according to the manufacturer’s instructions. For HLA-DR1 and HLA-DR2 screening, Micro SSP HLA typing trays specific for HLA-DR1 or HLA-DR2 (One Lambda, Canoga Park, CA) were used.

Antigen processing and presentation assays. THP-1 cells or human MDMs were cultured in 96-well flat-bottom plates (1.5 x 105 cells/well) in complete RPMI 1640 medium containing 10% pooled human serum. Both THP-1 cells and human MDMs were infected with M. tuberculosis at a multiplicity of infection ranging from 1 to 40. Bacteria were pelleted onto cells by centrifugation at 900 x g for 10 min at 37°C. Cells were incubated at 37°C for 10 min (providing a total pulse period of 20 min) and washed in ice-cold RPMI to remove extracellular bacteria. Prewarmed medium was added, and cells were incubated at 37°C for up to 4 h. Cells were fixed with 1% paraformaldehyde and washed.

T hybridoma cells (F9A6; 106) or M. tuberculosis-specific CD4+ T cells (106) were added to each well. All lymphocytes were infected with 100,000 (100 μl) harvested. Uptake of heat-killed live M. tuberculosis by THP-1 cells and MDMs was assessed by identification of acid-fast bacilli with Ziehl-Neelsen staining of cells. Three hundred cells were analyzed to determine the number of cells that had phagocytosed M. tuberculosis. We also determined phagocytosis by flow cytometry by incubating MDMs and THP-1 cells with FLUOS-labeled heat-killed or live M. tuberculosis as described previously (27).

In experiments with F9A6 T hybridoma cells, H-2 in the supernatants was measured using an IL-2-dependent CTLL-2 cell line as previously described (17). Proliferation of CTLL-2 cells was monitored by addition of Alamar blue (Trek Diagnostics, Westlake, OH) as an indicator dye and measured as the difference between absorbances at 550 and 595 nm after 24 h. The results presented are the mean of triplicate wells. In experiments with primary T cells, IFN-γ in supernatants was measured by enzyme-linked immunosorbent assay (ELISA) for IFN-γ antibodies (Endogen, Woburn, MA), and absorbance was read on a microplate reader (Labsystems Multiskan MCC/340, Labsystems, Finland) at 450 nm.

Subcellular fractionation of macropores for biochemical analysis and T-cell assay. THP-1 cells or MDMs were replated in six-well plates at 1.5 x 105 cells/well. Four plates were used for each fractionation. Cells were pulsed with medium containing heat-killed M. tuberculosis (multiplicity of infection, 40) for 20 min as described above, followed by various chase incubations. Cells were washed, detached by scraping and resuspended in 1 ml of homogenization buffer (0.25 M sucrose and 10 mM HEPES, pH 7.2). Samples were homogenized in a Dounce homogenizer (Wheaton) to obtain 40 to 50% homogenate. Intact cells and nuclei were removed by three consecutive spins at 2000 x g for 5 min at 4°C. The supernatant (containing phagosomes) was collected, layered on 9 ml of 27% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) in homogenization buffer and centrifuged in a T-865.1 fixed-angle rotor (Sorval, New York, CO) at 4°C for 60 min at 32,000 x g. Gradients were manually fractionated from the top into 30 fractions (333 μl each). Each fraction was divided into replicate aliquots of 10, 30, or 50 μl in 96-well plates and stored frozen at −80°C.

Plasma membrane was labeled before homogenization with sulfo avidin-biotin (Pierce, Rockford, IL). Cells were incubated with 0.5 μg/ml sulfo avidin-biotin for 4 h at 30°C, washed and incubated with 10 μg/ml streptavidin-fluorescein isothiocyanate (BD Bioscience, San Jose, CA) at 4°C for 30 min. Fractions (50 μl) were transferred to 96-well, clear-bottom black plates (Costar, Cambridge, MA) and analyzed on a Spectra Fluor Plus fluorometer (Tecan, United Kingdom). β-Hexosaminidase activity was measured by colorimetric assay. Fractions (50 μl) were incubated with 150 μl β-hexosaminidase buffer containing 0.1 M morpho-
line ethanesulfonic acid (MES), 0.2% Triton X-100, pH 6.5, and 50 μl of 1.13 mg/ml p-nitrophenyl-acetyl-D-glucosaminidase (Sigma-Aldrich). After 90 min at 37°C the reaction was stopped by addition of 0.5 M glycine, pH 10, and the optical density was determined at 405 nm.

To identify phagosomes containing fluorescent bacteria, fractions (50 μl) from cells infected with fluorescein-labeled M. tuberculosis were transferred into 96-well clear-bottom black plates (Costar) and analyzed on a Spectra Fluor Plus fluorimeter (Tecan). For T-cell analysis of Percoll gradient fractions, fractions were frozen and thawed once. Standard medium and F9A6 T hybridoma cells or M. tuberculosis-specific CD4⁺ T cells (10⁵ cells/well) were added to the individual fractions to a final volume of 200 μl. In wells containing polyclonal autologous M. tuberculosis-specific CD4⁺ T cells, 2 μg/ml of anti-human CD28 antibody from eBioscences (San Diego, CA) was added. After 24 h supernatants were harvested, and IL-2 or IFN-γ production was assessed. Wells containing 27% Percoll in homogenization buffer were used as blank controls. For identification of MIIC, noninfected THP-1 cells were incubated with soluble Ag 85B (5 μg/ml) for 1 h prior to fractionation. Fractions were frozen and thawed and then assessed for the presence of the M. tuberculosis Ag 85B (96-111)–DR1 complexes by the T-cell assay with F9A6 T hybridoma cells.

Preparation of phagosomes for flow organellometry. THP-1 cells or MDMs were plated in six-well plates at 1.5 × 10⁶ cells/well. Four plates were used for each condition. Cells were pulsed with fluorescein-labeled heat-killed M. tuberculosis (multiplicity of infection, 40) for 20 min as described above and chased for an additional 30 min. Cells were washed, detached by scraping, and resuspended in homogenization buffer. Cells were homogenized, and intact cells and nuclei removed by centrifugation. Phagosomes were pelleted from the supernatant at 500 × g at 4°C for 15 min and resuspended in 1 ml homogenization buffer. Resuspended phagosomes were isolated on 27% Percoll density gradients. Fractions previously identified to contain phagosomes (see above) were collected and combined with equal volumes of 2% paraformaldehyde for 10 min. The suspen-
sion was mixed with an equal volume of 0.4 M lysine in phosphate-buffered saline, and phagosomes were washed twice by pelleting and then resuspended in 0.5 ml phosphate-buffered saline (29). Phagosomes were stained with antibodies and isotype controls in 96-well plates using a buffer containing saponin to allow access to luminal epitopes and analyzed using a Coulter EPICS Elite ESP instrument (Beckman Coulter, San Diego, CA).

Antibodies and reagents. The following antibodies were used for staining phagosomes: Cy5-conjugated mouse anti-human LAMP-1 monoclonal antibody, Cy5-conjugated mouse anti-HLA-DR monoclonal antibody, Cy5-conjugated mouse immunoglobulin G2b isotype control, Cy5-conjugated mouse immunoglobulin G1 isotype control (BD Biosciences). Recombinant Ag 85B of M. tuberculosis was obtained from David Lakey, synthetic peptide 96-111 was obtained from Mimotopes Ltd. (Melbourne, Australia), and anti-human CD28 antibody was obtained from eBiosciences (San Diego, CA).

RESULTS

Characterization of M. tuberculosis processing and presentation. Processing of M. tuberculosis was analyzed in THP-1 cells and MDMs using an M. tuberculosis-specific T-cell hybridoma, F9A6. The F9A6 T-cell hybridoma was specific for M. tuberculosis Ag 85B (96-111) presented by HLA-DR1 and responded to THP-1 cells (which express HLA-DR1) and MDMs from HLA-DR1-positive donors following incubation with heat-killed M. tuberculosis (Fig. 1A). No response was observed in the absence of M. tuberculosis or when HLA-DR1/H11002 MDMs were incubated with heat-killed M. tuberculosis, confirming the antigen specificity and HLA-DR1 restriction of the F9A6 T-cell hybridoma.

To determine the kinetics of processing of M. tuberculosis antigens, F9A6 T hybridoma cells and M. tuberculosis-specific CD4+ T cells were used to detect peptide–MHC-II complexes. While the F9A6 T-cell hybridoma allowed analysis of processing of a specific M. tuberculosis antigen, polyclonal autologous M. tuberculosis-specific CD4+ T cells provided an assessment of a broad spectrum of M. tuberculosis antigens. THP-1 cells and MDMs were pulsed for 20 min with heat-killed M. tuberculosis or live M. tuberculosis at a multiplicity of infection of 40 and chased for various lengths of time for up to 4 h (Fig. 1B to D).

Processing of heat-killed M. tuberculosis was initiated rapidly in both THP-1 cells and MDMs, and M. tuberculosis Ag 85B...
(96-111)–DR1 complexes were expressed at high levels on the cell surface by 60 min (Fig. 1B and C). *M. tuberculosis*-specific CD4+ T-cell responses to MDMs incubated with heat-killed *M. tuberculosis* was also observed by 60 min (Fig. 1D). In contrast, processing of live *M. tuberculosis* by both THP-1 cells and MDMs was poor, with weak T-cell responses even at a chase incubation of 240 min (Fig. 1B and C). However, after 18 h of chase, live *M. tuberculosis* processing by both THP-1 cells and MDMs was similar to heat-killed *M. tuberculosis* (data only shown for THP-1 cells in Fig. 1B). It should be noted that THP-1 cells and MDMs were equally efficient in phagocytosis of live and heat-killed *M. tuberculosis* as assessed by identification of acid-fast bacilli with Ziehl-Neelsen staining of cells and analysis of cells incubated with FLUOS-labeled heat-killed and live *M. tuberculosis* by flow cytometry. In conclusion, human macrophages process and present heat-killed *M. tuberculosis* more rapidly and efficiently than live *M. tuberculosis*.

Subcellular fractionation of THP-1 cells and MDMs incubated with *M. tuberculosis*. Studies of phagosomal antigen processing require separation of phagosomes and other subcellular organelles for subsequent analysis. Following various treatments, THP-1 cells and MDMs were fractionated on Percoll density gradients. The distribution of organelles was carefully determined as previously described with a few modifications (27). To identify phagosomes, cells were incubated with FLUOS-labeled *M. tuberculosis*. To identify the plasma membrane, cells were incubated with sulfo-NHS-LC-biotin, followed by incubation with streptavidin-fluorescein at 4°C. To identify MIIC vesicles, cells were incubated with soluble Ag 85B to label *M. tuberculosis* Ag 85B (96-111)–DR1 complexes. Cells were homogenized, and phagosomes and other organelles were separated on 27% Percoll density gradients.

Phagosomes consistently appeared in fractions 27 to 30 in the high-density region of 27% Percoll gradients (Fig. 2A), whereas plasma membrane-associated fluorescence was found in fractions 9 to 12 in the low-density region (Fig. 2B). β-Hexosaminidase, a lysosomal enzyme, was primarily found in high-density phagolysosomal/lysosomal fractions (Fig. 2C, fractions 25 to 27). Similar levels of β-hexosaminidase were found in fractions derived from cells incubated with either heat-killed or live *M. tuberculosis*. FA96 T hybridoma cells detected *M. tuberculosis* Ag 85B (96-111)–DR1 complexes in MIIC endocytic vesicles in fractions 18 to 27 and sometimes on the plasma membrane in fractions 10 and 11 (Fig. 2D). The same results were obtained with fractionation of MDMs (data not shown).

Analysis of subcellular fractions for *M. tuberculosis* peptide–MHC-II complexes using a T-cell assay. To determine the site(s) where *M. tuberculosis* peptide–MHC-II complexes were formed during *M. tuberculosis* processing, we used a technique previously

(A), 30 min (B), and 2 h (C) before homogenization. Subcellular organelles were separated on 27% Percoll density gradients. *M. tuberculosis* Ag 85B (96-111)–DR1 complexes were detected using FA96 T hybridoma cells. Culture supernatants were harvested after 24 h and IL-2 production was measured by using a CTLL-2 proliferation assay. Diagrams at the top summarize the positions of different compartments in the Percoll gradients (Fig. 2). Data are representative of four independent experiments.
developed to detect peptide–MHC-II complexes in latex bead and *M. tuberculosis* phagosomes and other organelles in murine cells (12, 26, 27). This approach uses subcellular fractionation to isolate organelle membranes, which are disrupted by freezing and thawing to expose the luminal antigen-presenting domain of MHC-II molecules and then probed for peptide–MHC-II complexes using T cells.

High levels of peptide–MHC-II complexes appeared on the cell surface following a 60-min chase with heat-killed *M. tuberculosis* (Fig. 1). Therefore, in order to determine where peptide–MHC-II complexes are formed intracellularly, chase periods of less than 60 min are needed. THP-1 cells and MDMs were pulsed for 20 min with heat-killed *M. tuberculosis*, chased for either 0, 30 or 120 min and fractionated on 27% Percoll gradients. THP-1-derived fractions were incubated with F9A6 T hybridoma cells, and detection of *M. tuberculosis* Ag 85B (96-111)–DR1 complexes was monitored by IL-2 secretion (Fig. 3). MDM-derived fractions were incubated with *M. tuberculosis*-specific CD4+ T cells, and detection of *M. tuberculosis* peptide–MHC-II complexes was monitored by IFN-γ secretion (Fig. 4). We were unable to analyze subcellular fractions of macrophages infected with live *M. tuberculosis* for up to 6 h due to insufficient signal for T-cell assays. Fractionation of cells infected with live *M. tuberculosis* overnight was not technically possible.

When THP-1 cells were pulsed with heat-killed *M. tuberculosis* for 20 min with no chase incubation, *M. tuberculosis* Ag 85B (96-111)–DR1 complexes were detected primarily in phagosomes (fractions 26 to 28) with little expression on the plasma membrane (Fig. 3A). After a 20-min pulse and 30-min chase, *M. tuberculosis* Ag 85B (96-111)–DR1 complexes were detected in both phagosomes (fractions 26–29) and MIIC fractions (fractions 18 to 27) as well as on the plasma membrane (fractions 9 to 14, Fig. 3B). When the chase period was increased to 120 min, *M. tuberculosis* Ag 85B (96 to 111)–DR1 complexes were detected primarily in plasma membrane fractions with some complexes still present in MIIC compartments (Fig. 3C). When 0.5 μM *M. tuberculosis* Ag 85B (96-111) peptide was added to the fractions during the T-cell assay, F9A6 cells responded to all fractions containing MHC-II, providing a positive control and confirming the ability of F9A6 T cells to respond to all fractions where *M. tuberculosis* Ag 85B (96-111)–DR1 complexes were present (data not shown).

Thus, in THP-1 cells, *M. tuberculosis* Ag 85B (96-111)–DR1 complexes were initially formed in *M. tuberculosis* phagosomes (Fig. 3A). The presence of complexes in the MIIC compartment at the 30-min chase point (Fig. 3B) suggests either that the MIIC compartment receives phagosome-derived antigen fragments for generation of *M. tuberculosis* Ag 85B (96-111)–DR1 complexes within the MIIC compartment or that complexes formed in the phagosomes traffic through the MIIC compartment en route to the plasma membrane.

When MDMs were pulsed with heat-killed *M. tuberculosis* specific CD4+ T cells. Culture supernatants were harvested after 24 h, and IFN-γ production was measured by ELISA. Diagrams at the top summarize the position of different compartments in the Percoll gradients. Data are representative of three independent experiments.
for 20 min without chase, *M. tuberculosis* peptide–MHC-II complexes appeared in the MIIC compartment (fractions 21 to 27) with little expression on plasma membrane (Fig. 4A). As the chase period was increased to 30 and 120 min, *M. tuberculosis* peptide–MHC-II complexes in MIIC declined and complexes increased in the plasma membrane fractions (Fig. 4B and C). Polyclonal *M. tuberculosis*-specific T cells did not respond to fractions from MHC-II mismatched donors. We conclude that in MDMs, *M. tuberculosis* peptide–MHC-II complexes are formed in the MIIC compartment that receives phagosome-derived antigen fragments.

Assessment of LAMP-1 and MHC-II expression in heat-killed *M. tuberculosis* phagosomes isolated from THP-1 cells and MDMs by flow organellometry. Since MDM phagosomes differed from THP-1 phagosomes in the formation of *M. tuberculosis* peptide complexes, we analyzed heat-killed *M. tuberculosis* phagosomes from both THP-1 cells and MDMs by flow organellometry. THP-1 cells and MDMs were incubated with fluorescein-labeled heat-killed *M. tuberculosis* for 20 min, washed, and chased for 30 min prior to homogenization. Fluorescein-labeled bacteria were used to prepare phagosomes to enable identification of phagosomes during flow organellometry by gating on both scatter properties and fluorescein signal. Phagosomes were isolated on 27% Percoll gradients and subsequently fixed in paraformaldehyde to maintain the structural integrity of the phagosomes. All staining with antibodies was done in a buffer containing saponin to allow access to luminal epitopes (27, 29). In our analysis of phagosomes we have subtracted background staining from antibody staining to determine the percentage of phagosomes that stained positive for different markers.

Acquisition of LAMP-1 was used as a marker for phagosome maturation (Fig. 5). After a 30-min chase incubation, approximately 66% (±9%) of the heat-killed *M. tuberculosis* phagosomes isolated from THP-1 cells showed positive staining for LAMP-1 with a mean fluorescence value (MFV) of 78 ± 8 (Fig. 5B). In contrast, only 17% (±7%) of phagosomes isolated from MDMs were positive for LAMP-1 (P = 0.002, unpaired *t* test, Sigma Stat) (Fig. 5F) with an MFV of 49 ± 6 (P = 0.006). Phagosomes were also evaluated for expression of MHC-II. In phagosomes isolated from THP-1 cells, around 57% (±13%) of phagosomes showed positive staining for MHC-II with an MFV of 75 ± 11. Although 50% (±9%) of phagosomes isolated from MDMs were positive for MHC-II staining, the MFV was 45 ± 11 (P = 0.77, unpaired *t* test) (MFV P = 0.027). We conclude that phagosomes from both THP-1 cells and MDMs acquired LAMP-1 and MHC-II but that THP-1 phagosomes had significantly higher levels of both LAMP-1 and MHC-II. These differences could potentially explain why THP-1 phagosomes supported formation of *M. tuberculosis* peptide–MHC-II complexes while MDM phagosomes did not.

**DISCUSSION**

We have previously shown that *M. tuberculosis* phagosomes generated in murine macrophages are fully competent antigen processing organelles that can mediate the formation of peptide–MHC-II complexes (25, 27). In our current studies we have extended our observations to human macrophages by analyzing *M. tuberculosis* processing in the human macrophage cell line THP-1 and in monocyte-derived macrophages for presentation to *M. tuberculosis* Ag 85B-specific, DR1-restricted
Processing of heat-killed *M. tuberculosis* for presentation to T cells (Fig. 1B to D) was initiated rapidly in both THP-1 cells and MDMs, and complexes could be observed on the cell surface by 60 min. In contrast, processing of live *M. tuberculosis* was poor and few complexes appeared on the cell surface even after prolonged chase incubations. However, after 18 h of chase, live *M. tuberculosis* processing was similar to that of heat-killed *M. tuberculosis* (Fig. 1B), indicating that *M. tuberculosis* viability influences the process involved in early events of antigen processing.

To determine if live and heat-killed *M. tuberculosis* differentially regulated MHC-II levels on THP-1 cells and MDMs, we assessed MHC-II surface levels on MDMs and THP-1 cells infected with heat-killed or live *M. tuberculosis* at an early time point (2 h) and at a late time point (18 h). While no differences were seen at the early time point, overnight incubation of cells with live *M. tuberculosis* resulted in decreased surface MHC-II (data not shown). Therefore, levels of surface MHC-II do not correlate with the differences in processing of heat-killed and live *M. tuberculosis* at early or late time points.

Pancholi et al. (21) have previously reported good antigen processing and presentation in macrophages infected for 2 days with live BCG and poor antigen presentation in macrophages infected for 4 days (chronic infection). While our results (18 h time point) are similar to what they found on day 2, our studies have focused on early events in antigen processing and presentation. Since live *M. tuberculosis* can inhibit phagosome maturation, our observations suggest that live *M. tuberculosis* may be able to inhibit both phagosome maturation and MHC-II antigen processing. However, phagosomal processing of *M. tuberculosis* Ag 85B in murine macrophages is modulated independently of mycobacterial viability and phagosome maturation (25).

To analyze the intracellular sites of *M. tuberculosis* peptide–MHC-II complex formation, such as MIIC and *M. tuberculosis* phagosomes, subcellular fractionation was coupled with *M. tuberculosis*-specific T-cell assays or flow organellometry. Plasma membrane and phagosome fractions were well separated on 27% Percoll gradients (Fig. 2), and no significant plasma membrane contamination of the phagosomal fractions was observed (no fluorescein isothiocyanate signal in phagosomal fractions, Fig. 2B). Since approximately 60% of total MHC-II is found on the plasma membrane in murine macrophages (13), contamination of phagosomes with plasma membrane would have interfered with evaluation of phagosomal MHC-II levels. The distribution of phagosomes and MIIC did overlap on 27% Percoll gradients (Fig. 2). Since MIIC contains intracellular MHC-II molecules, in some experiments where phagosomes were isolated for evaluation of MHC-II levels by flow organellometry, differential centrifugation to separate phagosomes from smaller membrane structures, e.g., MIIC, was carried out prior to fractionation on 27% Percoll gradients (27). This approach provided us with pure preparations of *M. tuberculosis* phagosomes for our flow organellometry analyses.

To determine where *M. tuberculosis* peptide–MHC-II complexes were formed intracellularly, macrophages were incubated with heat-killed *M. tuberculosis* for various periods and fractionated on Percoll density gradients. Fractionation of THP-1 cells and MDMs infected with live *M. tuberculosis* was unsuccessful due to insufficient signal for T-cell assays. In THP-1 fractions, *M. tuberculosis* Ag 85B (96-11)-DRI complexes appeared in phagosomes soon after pulse incubation with heat-killed *M. tuberculosis* (20 min) (Fig. 3A). Subsequently, complexes were additionally found in MIIC and plasma membrane fractions (Fig. 3B and C). These observations differ from those seen in murine macrophages pulsed with heat-killed *M. tuberculosis*, where *M. tuberculosis* Ag 85B peptide–MHC-II complexes were detected in phagosomes and plasma membrane fractions but not in MIIC fractions. The presence of *M. tuberculosis* Ag 85B (96-11)-DRI complexes in the MIIC fractions suggests that peptide–MHC-II complexes were either additionally generated in these compartments following export of *M. tuberculosis* protein fragments from the phagosome to the MIIC compartment (either directly or indirectly) or that peptide–MHC-II complexes formed in phagosomes trafficked via the MIIC en route to the plasma membrane.

In MDM fractions, no *M. tuberculosis* peptide–MHC-II complexes were ever observed in phagosomes (Fig. 4). We consistently saw complexes in the MIIC and plasma membrane fractions. Therefore, in MDMs, *M. tuberculosis* peptide–MHC-II complexes are formed in MIIC following direct or indirect export of protein fragments from phagosomes to MIIC compartments. Several observations do support a role for endocytic compartments (e.g., MIIC) in the formation of complexes derived from phagocytic antigens. For example, export of Ag 85 in addition to other proteins and lipids, from the *M. tuberculosis* phagosome to other intracellular compartments, has been reported by several investigators (3, 20, 31, 35, 41). In addition, in murine macrophages infected with *Streptococcus pyogenes*, peptide–MHC-II complexes were consistently observed in endocytic compartments and not in phagosomes (9).

With polyclonal autologous CD4 T cells we are analyzing a broad spectrum of *M. tuberculosis* peptide–MHC-II complexes in MDMs. This approach is useful to generalize our observations of *M. tuberculosis* processing. Since antigens may differ in subcellular processing compartmentalization, this may explain the differences seen between THP-1 cells and MDMs. Alternatively, MDM phagosomes may be incapable of supporting the formation of *M. tuberculosis* peptide–MHC-II complexes and differ from THP-1 phagosomes in their MHC-II levels and phenotype.

In order to analyze MDM and THP-1 phagosomes we carried out flow organellometry of phagosomes containing fluorescein-labeled heat-killed *M. tuberculosis*. Most of the THP-1 phagosomes were LAMP-1 positive compared to MDM phagosomes, and the levels of LAMP-1 in THP-1 phagosomes were also higher than that seen in MDM phagosomes. Since THP-1 cells were activated with IFN-γ to increase MHC-II levels (to facilitate T-cell assays) and IFN-γ is known to promote phagosome maturation (2, 18, 32, 38), we do not know whether the difference in LAMP-1 levels is due to IFN-γ activation of THP-1 cells or due to more intrinsic differences between the two cell types.

Within the time frame of active antigen processing, similar percentages of heat-killed *M. tuberculosis* phagosomes from both THP-1 cells and MDMs acquired MHC-II molecules that could
potentially bind peptides derived from \textit{M. tuberculosis} antigens. However, the levels of MHC-II in MDM phagosomes were lower than those of THP-1 phagosomes despite the fact that IFN-γ-activated THP-1 cells had surface MHC-II levels that were only 80% ± 8% of that seen in MDMs. Since MDM phagosomes appear to be different from THP-1 phagosomes and acquire fewer MHC-II molecules (Fig. 5), it is possible that MDM phagosomes are incapable of supporting the formation of \textit{M. tuberculosis} peptide–MHC-II complexes.

There are other alternative explanations for the differences in processing between IFN-γ-activated THP-1 cells and MDMs. IFN-γ activation could have induced the expression of different receptors on THP-1 cells and altered the mode of entry of \textit{M. tuberculosis} and the subsequent processing events. IFN-γ could have also altered trafficking events in THP-1 cells, resulting in the differences that we have observed.

In conclusion, we have observed that human macrophage process and present heat-killed \textit{M. tuberculosis} far more rapidly than live \textit{M. tuberculosis}. While \textit{M. tuberculosis} phagosomes can mediate the formation of peptide–MHC-II complexes in THP-1 cells, it is the MII compartment that receives phagosome-derived antigen fragments for generation of \textit{M. tuberculosis} peptide–MHC-II complexes in MDMs. Future studies will help determine whether the maturation status of the phagosome dictates its ability to generate peptide–MHC-II complexes in human macrophages.

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